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# A baculovirus vector derived from immediately early gene promoter of *Autographa californica* nuclear polyhedrosis virus

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**Abstract**

A transfer vector was constructed in which the neomycin resistance (neo) gene was under the control of a copy of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) IE1 gene promoter at the p10 locus. After cotransfection of *Spodoptera frugiperda* (Sf9) cells with the transfer vector and infectious AcMNPV DNA, the recombinant virus-containing neo gene was selected by serial passage of the mixed progenies from cotransfection. This was done at low MOI in the presence of G418 in growth medium and was followed by limited dilution. RNA dot hybridization showed that the neo gene was transcribed from immediately early phase to very late phase, in infected Sf9 cells. These results demonstrate that a new baculovirus vector system had been constructed in infected cells. Furthermore, a new method for selection of positive recombinant baculovirus had been developed.

*Keywords:* Baculovirus; Transfer vector; Neomycin gene; IE1 promoter; Selection method

## 1. Introduction

Baculoviruses are arthropod-specific viruses, studied extensively for their potential as insecticidal agents (Entwistle and Evans, 1985) and as expression vectors for prokaryotic and eukaryotic proteins (Luckow and Summers, 1988; Miller, 1989; O'Reilly et al., 1992; King and Possee, 1992). Baculovirus expression vector is a recombinant virus in which the coding sequence for a desired foreign protein has been placed under the control of the promoter of a viral gene. Current baculovirus expression vectors are based on the exploitation of the polyhedrin and p10 promoters which are quite active during the very late phase of virus infection (Kelly and Lescott, 1981), and

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on findings that deletion of the polyhedrin or p10-coding sequence from the viral genome doesn't impair viral replication in vitro (Fraser et al., 1983; Vlak et al., 1990; Blissard and Rohrmann, 1990). Hence, both genes can be replaced by foreign genes for high level expression of foreign protein, under the control of either the polyhedrin or p10 promoter. The promoter of basic protein of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) has also been used for protein expression (Hill-Perkins and Possee, 1990). Further data showed that use of the basic protein promoter is more favorable for high yield expression of foreign protein than that of prototype polyhedrin or p10 protein promoter in the baculovirus expression system (Bonning et al., 1994).

Baculoviruses have been used widely as a biological control agent, but the combination of a slow speed of effectiveness and narrow host range limit their application. The development of a baculovirus expression system makes it possible to enhance the virus pathogenicity to insect larvae by expressing protein with insecticidal activity. A number of attempts have been made to construct recombinant viruses by inserting various genes into the virus genome under the control of the polyhedrin promoter, which included genes coding for scorpion neurotoxins (Carbonell et al., 1988; Stewart et al., 1991); diuretic hormone (Maeda, 1989); juvenile hormone esterase (Hammock et al., 1990); mite toxin (Tomalski and Miller, 1991); and $\delta$-endotoxin gene of *Bacillus thuringiensis* (Merryweather et al., 1990; Martens et al., 1990; Qi et al., 1994). Among these, the mite toxin (Tomalski and Miller, 1991) and scorpion toxin (Stewart et al., 1991) showed a significant increase in speed of action.

In order to construct fast-acting recombinant baculovirus insecticide, investigation of viral promoters other than the polyhedrin and p10 is needed. When determining the promoter to be used for foreign gene expression in the baculovirus system, the factors to be considered vary according to the primary purpose of the recombinant. For rapid effects on pest insects exposed to recombinant viral insecticides, low level of expression early post infection may be more favorable than high level expression late. In contrast, for expression of proteins for pharmaceutical applications, yield is the prime consideration, although early production still offers an advantage with authenticity (e.g. correct glycosylation is required).

IE1 gene which encodes a trans-activator of delayed-early promoters (Guarino and Summers, 1987, 1986), is expressed very early in AcMNPV infected cells (Chisholm and Henner, 1988). IE1 gene is an essential gene which is required for late and very late gene expression. Theilmann and Stewart (1993) analyzed the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) IE1 gene product by using monoclonal antibodies, and found that there were multiple forms of IE1 product which have steady-state level increment throughout infection from early to very late phase.

In this report we establish a baculovirus vector based on AcMNPV IE1 gene promoter, the intention being that a recombinant virus-containing toxin gene derived from this vector, with early expression in infected cells, would show an improved speed of killing.

## 2. Materials and methods

### 2.1. Cells and virus

*Spodoptera frugiperda* cell line (Sf9) and wild type (wt) *Autographa californica* nuclear polyhedrosis virus E2 strain were kindly provided by Dr. Federici (University of California, Riverside). Monolayer cultures of Sf9 cells were maintained at 27°C in TNM-FH medium supplemented with 10% fetal bovine serum (FBS, Gibco). For infection, cells were inoculated with extracellular BV and rocked for 1 h at room temperature. The residual inoculum was removed and replaced by growth medium. When indicated, G418 (Geneticin, GIBCO Laboratories) was added to the growth medium.

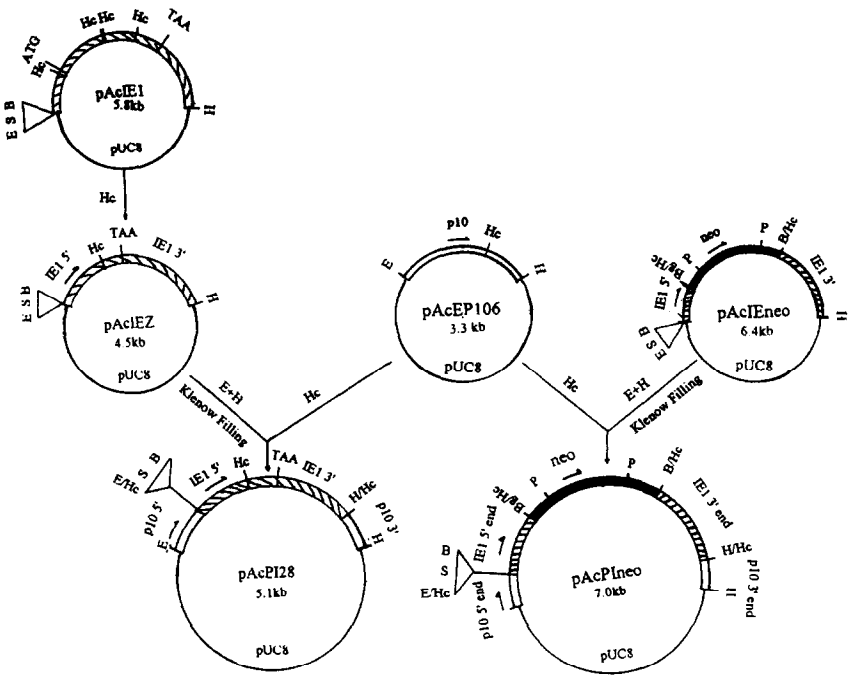


Fig. 1. Construction of the IE1 gene promoter transfer vector. A schematic representation of the procedures used to derive pAcPI28 and pAcPIneo. Thin lines represent the pUC vector. Solid box is the neo gene sequence, the open box is AcMNPV p10 gene sequence and the hatched box is AcMNPV IE1 gene sequence. Restriction enzyme sites: E, *Eco*RI; B, *Bam*HI; S, *Sma*I; H, *Hin*dIII; P, *Pst*I; Bs, *Bs*36I; Bg, *Bgl*II; Hc, *Hin*cII; Hc/Bg, *Hin*cII/*Bgl*II fusion; Hc/B, *Hin*cII/*Bam*HI fusion; Hc/E, *Hin*cII/*Eco*RI fusion; Hc/H, *Hin*cII/*Hin*dIII fusion.

### 2.2. Extraction and cloning of DNAs

The construct of transfer vectors used in this study is shown schematically in Fig. 1.

The preparation of plasmid DNA from *E. coli* and viral DNA from virions, digestion with restriction nucleases (Gibco), were done according to procedures by Maniatis and Summers and Smith (1987). The plasmid pAcEP106 was constructed from pAcHQ which contained the AcMNPV *Hin*dIII-Q fragment at the HindIII site of plasmid pUC8. pAcHQ was digested by *Eco*RI and self ligated to remove the 5′ upstream sequence of p10 gene, which included hr5 and p26 gene. The resulting plasmid was designated as pAcEP106 which has 600 bp insert with complete 5′ non-coding sequence and partial coding sequence of p10. A *Hin*cII site was located at 63 bp downstream from the translational start site of p10.

Plasmid pAcIE1 (provided by Jarvis et al., 1990) containing a cloned copy of AcMNPV IE1 gene (Guarino and Summers, 1986, 1987), had a *Hin*cII site located at 39 bp upstream of the translational start site and three other *Hin*cII sites in IE1 coding sequence. Plasmid pAcIE1 was completely digested with *Hin*cII and self-ligated to remove most of the coding sequence and leave the 5′ and 3′ noncoding sequence. The resulting plasmid was designated as pAcIEZ. pAcIEZ and was digested with *Eco*RI and *Hin*dIII. A 1.8 kb fragment containing IE1 promoter and a 3′ noncoding sequence was isolated, blunted by Klenow (Gibco) and then inserted into the *Hin*cII site of pAcEP106. Recombinant plasmid, in which the p10 gene has the same orientation as IE1 gene, was selected by using restriction endonuclease digestion and designated as pAcPI28. This vector contained a unique *Hin*cII site downstream of the IE1 promoter which can be used to clone a foreign gene.

pAcIEneo (also provided by Jarvis and Guarino) was used to construct a transfer vector containing neomycin resistance gene (neo) immediately downstream of IE1 promoter. This plasmid was constructed by inserting a Klenow-repaired *Bgl*II-*Bam*HI fragment- containing neo gene into pAcIE1 at *Hin*cII site located 39 bp upstream of the translational start site of IE1. pAcIEneo was digested with *Eco*RI and *Hin*dIII. A 3.7 kb fragment containing IE-neo was purified in agarose gel (Gene Clean, Promega), filled-in by Klenow and subcloned into pAcEP106 digested with *Hin*cII. Positive recombinant plasmid in which the p10 gene had the same orientation as IE1 gene was selected by restriction endonuclease digestion and designated as pAcPIneo. Plasmid pAcPIneo was equal to recombinant plasmid with the neo gene at *Hin*cII site of pAcPI28.

### 2.3. Co-transfection

Infectious DNA of wild type AcMNPV from BV and plasmid DNA were prepared as described (Summers and Smith, 1987). The monolayer culture of Sf9 cells ($2 \times 10^6$ cells) was cotransfected with virus DNA (1.0 $\mu$g) and plasmid DNA (20 $\mu$g) using calcium phosphate modified precipitation method as described by Summers and Smith (1987). After transfection, (4–7 days) the culture containing the mixed viral progenies was harvested, centrifuged and titrated.

### 2.4. Titration of virus infectivity

The infectivity of non-occluded virus (NOV) was determined by end-point dilution method (Summers and Smith, 1987) using the occurrence of polyhedra as an indicator of infection. The titer was expressed as $TCID_{50}$/ml. Another parallel 96 well culture was used to determine the proportion of recombinant viruses. To determine the titer of neo-containing recombinant virus, 2 mg/ml G418 was added .

### 2.5. Selection and purification of recombinant virus

The mixed viral progenies were then subjected to a limited dilution method. Briefly, cells were inoculated by mixed viral progenies with multiplicity of infection (MOI) of 0.05–0.1, kept for 1 h at room temperature, and then washed twice with fresh medium to remove uninfected particles. Fresh medium, containing 2.0 mg/ml G418 (Gibco) and 10% FBS, was added, and after 3–4 days, the medium was collected by centrifugation. To infect a fresh monolayer of cells, 1/1000 of this supernatant was used and the remnants were stored for titration. Subsequent passage inoculation used 1/1000 supernatant from previous infection. At each passage the viruses were harvested. After six passages,a culture of recombinant virus was obtained (Zhu et al., 1995).

The viral stock was diluted serially ($10^{-1}$–$10^{-8}$) and used to infect fresh Sf9 cells in a 96 well microtiter plate and G418 was added. After 7 days, the medium from the infected well with the highest dilution was taken as pure recombinant baculovirus and named vAcneo.

### 2.6. DNA analysis of recombinants

Total cellular DNA of purified recombinant and wild type AcMNPV were extracted by disrupting cells in lysis buffer (25 mM Tris-HCl, pH 8.0, 2.5% 2-mercaptothanol, 0.2% SDS, 50 mM EDTA) incubated with 250 $\mu$g/ml proteinase K (Promega) for 30 min at 37°C and then extracted twice with phenol/chloroform before ethanol precipitation. The DNA was resuspended in water and analyzed by Southern hybridization using neo gene as a probe.

### 2.7. Microscopy study of cell lysis

One million Sf9 cells were seeded in culture dishes and infected at MOI of 10 with wt AcMNPV and recombinant virus vAcneo. After 90 min of incubation the inoculum was removed and cells were washed with TNM-FH medium supplemented with 10% FBS. The cells were

covered with a thin agar overlayed with 1.5% agarose in the medium. At 96 h p.i., cells were examined by microscopy.

### 2.8. Isolation and hybridization of total cellular RNA

Total cellular RNA at different times post infection were extracted by King's method and subjected to dot hybridization(Sambrook et al., 1989). The probe of the *Pst*I fragment of pAcIEneo was gel purified and labeled with $\alpha$-$^{32}$P-dATP by random primer method (Promega).

## 3. Results

### 3.1. Construction of transfer vector

Since the IE1 gene is a functional gene, it is unlikely that replacing the IE1 gene of AcNPV with a foreign gene would yield a viable recombinant virus. Attempts were made to duplicate the IE1 promoter in an alternative position of the viral genome, which would not affect virus replication. The suitable positions for insertion of a foreign gene were p10 and polyhedrin loci. Since our aim is to construct a more effective virus insecticide, we selected p10 locus to retain polyhedra, which is important for virus stability in an outside environment.

Because p10 disruption cannot produce visual CPE we cannot select the recombinant virus by the general method. We chose neo as a report gene which can also serve as a selection marker for recombinant baculovirus (Lerch and Friesen, 1993).

### 3.2. Generation and selection of recombinant virus

After co-transfection (4–7 days) of Sf9 cells with pAcPIneo and wt AcMNPV DNA, when polyhedra can be seen, the growth medium which had a mixture of parent virus and recombinant progeny virus was collected. This mixture was subjected to titration by end-point dilution method. To determine the proportion of recombinant viruses, 2 mg/ml G418 was added to 96 well as parallel assay. After 4–7 days, any infected wells (showing polyhedra) were scored as positive and the $TCID_{50}$ value was calculated using Reed and Muech's method as described by Summers and Smith (1987). The result of recombinant frequency was about 1% which is comparable to standard polyhedrin desired transfer vectors.

As shown previously (Lerch and Friesen, 1993), neo under AcMNPV p35 gene promoter can serve as a positive selector for AcMNPV. To confirm whether the neo gene under IE1 promoter can do so, we inoculated Sf9 cells with the co-transfection mixture at a MOI of 0.05 and treated them with 2 mg/ml G418. By determining $TCID_{50}$ value with G418 or not, we found the proportion of recombinant virus increased from 1 to 10% after 7 days, which indicated that the neo gene could be expressed under the control of a IE1 gene promoter providing an advantage in selection of recombinant viruses when treated with G418.

The virus mixture was passaged at low MOI and treated with 2 mg/ml G418 several times. After 4 times, it was found that the titer of virus with G418 treatment (Fig. 2) was almost equal to the titer without G418 treatment which means that almost all of the viruses in the growth medium were recombinant viruses. Fig. 2 also showed that the titer of the recombinant virus containing neo gene increased with no detectable increment of the total titer, which indicated that there were an increment in the proportion of recombinant viruses at each passage.

### 3.3. Release of polyhedra

To study the infection of recombinant virus and wild type (wt) AcMNPV under the microscope, purified recombinant virus vAcneo and wt-AcMNPV were used to inoculate Sf9 cells at MOI of 20. Infection of wt-AcMNPV led to high level of polyhedra in the culture medium at 96 h p.i., whereas polyhedra were retained inside the nucleus of infected cells during the infection of vAcneo, (Fig. 3).

### 3.4. DNA analysis of recombinant virus

The DNA of the recombinant virus was analyzed by restriction endonuclease analysis with *Hin*dIII and compared with wild type AcMNPV (Fig. 4). The 2.1 kb *Hin*dIII-Q fragment of wild type AcMNPV was not present in the recombinant virus due to the insertion of IE1-neo construct in this region. A fragment of about 5.8 kb was excised correctly from recombinant vAcneo by *Hin*dIII. Southern blot, using neo as a probe, confirmed the proper identity.

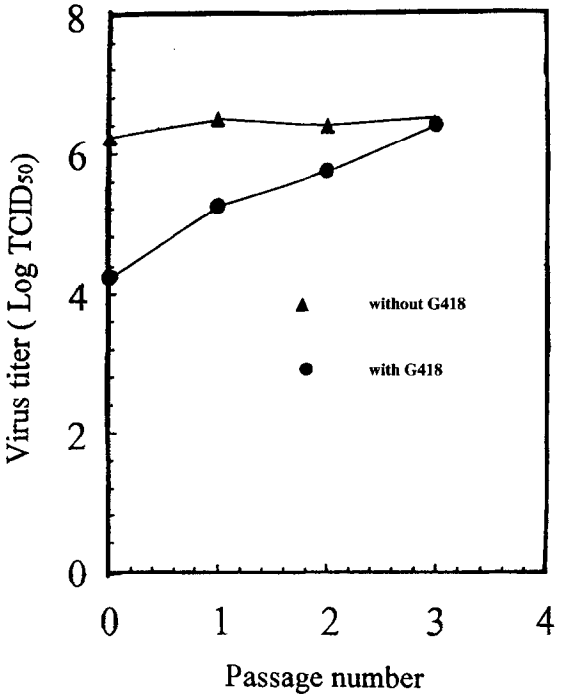


Fig. 2. Selective amplification of neo-containing recombinant viruses by serial passage. Sf9 cells ($2 \times 10^6$ cells) were inoculated with mixed progeny from cotransfection at 0.1 MOI, and covered with 3 ml growth medium containing G418 (2 mg/ml). The medium was collected at 72 h post infection and 3 $\mu$l was used to inoculate a subsequent culture ($2 \times 10^6$ cells). The virus titer of the remaining medium was determined by $TCID_{50}$ assay in two parallel tests (one with G418 added to medium, another not). The yield of total viruses (without G418, ▲) and recombinant viruses (with G418, ●) is shown for the four successive infections using a log scale.

### 3.5. RNA dot hybridization

The neo transcription was quantitated with RNA dot hybridization assay. To analyze the amount of mRNA produced by recombinant virus in Sf9 cells, total cellular RNA at different time post infection was extracted, transfered to NC membrane and hybridized using pAcIEneo *Pst*I 800 bp fragment as a probe. The result showed that the expression of neo gene started at 2 h p. i. and continued until 48 h p.i. (Fig. 5).

## 4. Discussion

A recombinant virus containing neo gene placed downstream of IE1 and p10 gene promoters was successfully isolated. The recombinant virus displayed no visible difference from wt-AcMNPV, and cannot be selected by traditional selection methods. Lerch and Friesen (1993) showed that the neo gene under AcMNPV p35 gene promoter provided dominant selection of recombinant baculovirus. Based on their result, we found that neo gene under IE1 promoter could also be used as an effective selection marker. By serially passaging the mixed progenies from co-transfection at very low MOI (not as Lerch by blind passage) under G418 selection, the viruses containing neo gene were selectively amplified to a high proportion. After several times serial passage, the proportion of recombinant virus was greater than 90%. The culture medium of the fourth passage was diluted serially ($10^{-1}$–$10^{-8}$) to infect Sf9 cells cultured in a 96 well plate under G418 selection. The culture medium from infected well with highest dilution was collected and regarded as purified homogenecity.

In fact, homologous recombination may take place at either p10 or IE1 locus in AcMNPV genome. Since IE1 gene is an essential gene, it will not produce a viable recombinant virus if recombination take place at IE1 locus. We presumed that the resulted recombinant virus must have IE1-neo integrated at p10 locus. Southern blot hybridization confirmed our presumption.

Consequently, the recombinant virus must have impaired the p10 gene, so that the release of


Fig. 3. Images of *S. frugiperda* cell cultures infected with wt AcMNPV (A), vAcneo (B) at 96 h p.i.

polyhedra will be blocked in infected Sf9 cells (William et al., 1989; Vlak et al., 1990). By microscopic examination, we found that release of polyhedra of vAcneo infection was blocked. Passaging vAcneo at MOI of 20 several times without G418 and examining by microscope after each passage, no polyhedra was found in medium (Fig. 4). This suggested that the resulted recombinant virus was sufficiently purified and also proved the selection method we developed was effective.

Selection of positive recombinant virus is a critical step for using the baculovirus expression system. The traditional method is very difficult for researchers who are not familiar with virology because several rounds of plaque purification are tedious and time-consuming. Many attempts to accelerate identification and purification steps have been reported but all have limitations (Zhu et al., 1995). We describe a selection method which was based on G418 selective amplification and limited dilution, and successfully selected the desired recombinant virus. This method can omit rounds of plaque purification and was both simple and effective in baculovirus expression system.

In transfer vector pAcPI28, the flanking sequence for homologous recombination is only about 600 bps which is shorter than other transfer vectors generally used. Thus, a question arises: Is the flanking sequence long enough for recombination? In general, the longer the flanking sequence, the higher the frequency of recombination. From our data, a total flanking sequence of 600 bp is sufficient to maintain a normal recombinant frequency. This result showed that most commonly used transfer vectors could be shortened to make DNA manipulation easier without interfering with the recombinant frequency. Further works were required to determine the shortest flankng sequence sufficient for normal recombi-

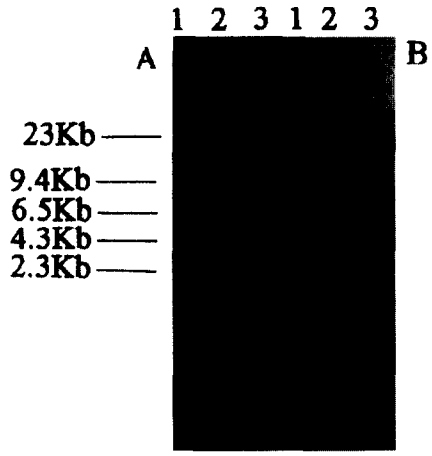


Fig. 4. Southern blot analysis of recombinant virus vAcneo. Total cellular DNA isolated from infected Sf9 cells were digested by *Hin*dIII, and the resulting fragments were runed in 0.7% agarose gel and blotted onto NC membrane. The membrane was hybridized with $^{32}$P-labelled, gel-purified 800-bp pIEneo *Pst*I fragment. A, EB staining; B, Radioautograph; Lane 1, lDNA/*Hin*dIII; Lane 2, vAcneo, Lane 3, wtAcMNPV.

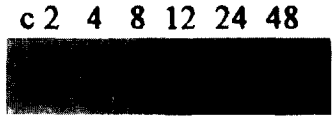


Fig. 5. RNA dot hybridization. Total RNA from infected cells were isolated, spotted to NC membrane and hybridized with $^{32}$P-labelled 800-bp pIEneo *Pst*I fragment. c, wt AcMNPV, 2–48 represents hours after infection of vAcneo.

nant frequency. By titrating virus under G418 selection or not, we developed a method of determining recombination frequency, which will be very useful in the study of factors affecting recombinant frequency.

A unique *Bsu*36I site in neo gene was introduced into vAcneo at p10 locus so that vAcneo DNA can be linearized by *Bsu*36I. When we use pAcPI28 to express other foreign proteins, there will be no selection marker for recombinant viruses. If we use linearized vAcneo DNA as helper virus DNA, the proportion of recombinant viruses increases to 30% (Kitts et al., 1990). The recombinant virus would be easy to select by dot hybridization.

G418 inhibits AcMNPV replication in Sf9 cells (Lerch and Friesen, 1993). The fact that vAcneo can normally replicate under G418 pressure suggested the neo gene was expressed in infected cells. From the result of RNA dot hybridization, we found that the neo gene was expressed from immediately early phase to very late phase. The expression during late phase increased to a higher level than expected. We presumed that the upstream p10 promoter may have some effect on IE1 transcription during late phase in infection. However, all the results demonstrated that a new baculovirus expression vector derived from the AcMNPV IE1 gene promoter has been successively constructed.

This new vector may have some advantage when producing protein requiring extensive post-translational modification, since the cell may be better equipped to carry out such processes at early infection. Jarvis et al. (1990) showed that *t*-PA expressed under IE1 promoter was processed faster and more efficiently in transformed Sf9 cells and addressed the fact the host cell secretary pathway was compressed during the late phase of virus infection.

The immediate early gene expression vector will also have an advantage when genetically engineering virus insecticide. It will now be feasible to express insect-specific toxin or hormone in infected insects earlier than would be possible with the polyhedrin or p10 expression system, possibly hastening the effect on the target insect.

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## References

Blissard, G.W. and Rohrmann, G.F. (1990) Baculovirus diversity and molecular biology. Annu. Rev. Entomol. 35, 127–155.

Bonning, B.C., Roelvink, P.W., Vlak, J.M., Possee, R.D. and Hammock, B.D. (1994) Superior expression of juvenile hormone esterase and $\beta$-galactosidase from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus compared to the p10 protein and polyhedrin promoters. J. Gen. Virol. 70, 187–202.

Carbonell, L.F., Hodge, M.R., Tomalski, M.D. and Miller, L.K. (1988) Synthesis of a gene coding for an insect-specific scorpion neurotoxins and attempts to express it using baculovirus vectors. Gene 73, 409–418.

Chisholm, G.E. and Henner, D.J. (1988) Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. J. Virol. 62, 3193–3200.

Entwistle, P.J. and Evans, P. (1985) Viral control. In G.A. Kerut and L.I. Gilbert. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol 7. Pergamon Press, New York, pp. 347–412.

Fraser, M.J., Smith, G.E. and Summers, M.D. (1983) Acquisition of host cell DNA sequences by baculoviruses: relationship between host DNA insertion and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. J. Virol. 47, 287–300.

Guarino, L.A. and Summers, M.D. (1986) Fuctional mapping of a trans-activating gene required for expression of baculovirus delayed-early gene. J. Virol. 57, 563–571.

Guarino, L.A. and Summers, M.D. (1987) Nucleotide sequence and temporal expression of a baculovirus regulatory gene. J. Virol. 61, 2091–2099.

Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N. and Maeda, S. (1990) Expression and effects of the juvenile hormone esterase in baculovirus vector. Nature 344, 458–461.

Hill-Perkins, M.S. and Possee, R.D. (1990) A baculovirus expression vector derived from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus. J. Gen. Virol. 71, 971–976.

Jarvis, D.L., Fleming, J.G.W., Kovacs, G.R., Summers, M.D. and Guarino, L.A. (1990) Use of Early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed Lepidopteran cells. Bio/Technol. 8, 950–955.

Kelly, D.C. and Lescott, T. (1981) Baculovirus replication: protein synthesis in *Spodoptera frugiperda* cells infected with Trichoplusia in nuclear polyhedrosis virus. Microbiology 4, 35–57.

Kitts, P.A., Ayers, M.D. and Possee, R.D. (1990) Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vector. Nucleic Acids Res. 18, 5667–5672.

King, L.A. and Possee, R.D. (1992) The Baculovirus Expression System, A Laboratory Guide, Chapman Hall, London.

Lerch, R.A. and Friesen, P.D. (1993) The 35-kilodalton protein gene (p35) of *Autographa californica* nuclear polyhedrosis virus and the neomycin resistance gene provide dominant selection of recombinant baculoviruses. Nucleic Acids Res. 21, 1753–1760.

Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol. 6, 47–55.

Maeda, S. (1989) Increased insecticidal effect by a recombinant baculovirus carry a synthetic diruretic hormone. Biochem. Biophys. Res. Commun. 165, 1177–1183.

Martens, J.W.M., Hone, G., Zuidema, D., Van Lent, J.W.M., Visser, B. and Vlak, J.M. (1990) Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. Appl. Environ. Microbiol. 56, 2764–2770.

Merryweather, A.T., Weyer, U., Harris, M.P.G., Hirst, M., Booth, T. and Possee, R.D. (1990) Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. Kurstaki HD-73 delta endotoxin. J. Gen. Virol. 71, 4835–4638.

Miller, L.K. (1989) Insect baculoviruses: powerful gene expression vectors. Bio. Essays 11, 91–95.

O'Reilly, D., Miller, L.K. and Luckow, V.A. (1992) Baculovirus Expression Vectors: A Laboratory Manual. W.H. Freeman, New York.

Qi, Y.P., Huang, Y.X., Shen, Y., Tan, Y.P. and Wang, F.S. (1994) Expression of *Bacillus thuringiensis* d-endotoxin gene with recombinant baculovirus in insect cell. Chinese Science Bulletin 39, 424–429.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning; A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Summers, M.D. and Smith, G.E. (1987) A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agric. Exp. Station Bull. 1555, p. 1–57

Stewart, L.M.D., Hirst, M., Lopes-Ferber, M., Merryweather, A.T., Cayley, P.J. and Possee, R.D. (1991) Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature, 352, 85–88.

Theilmann, D.A. and Stewart, S. (1993) Analysis of the *orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus transactivators IE-1 and IE-2 using monoclonal antibodies. J. Gen. Virol. 74, 1819–1826.

Tomalski, M.D. and Miller, L.K. (1991) Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature 352, 82–85.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1990) Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-$\beta$-galactosidase fusion gene. J. Gen. Virol. 69, 765–776.

William, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989) A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion deletion mutants. J. Gen. Virol. 70, 187–202

Zhu, F.X., Wang, F.S. and Qi, Y.P. (1995) Development of selection methods for positive recombinant baculoviruses. Prog. Biochem. Biophys. 22, 31–35 (in Chinese).